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Methods assessment of self-tanning of rapeseed and sunflower meal fractions enriched in proteins and phenolic compounds using *in vitro* measurement of protein rumen degradability☆ ☆☆

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Abstract – Two protein tanning methods were evaluated to contribute to the withdrawal of formaldehyde as a tanning agent of meals for feeding ruminants. The experimental materials were two fractions of rapeseed and sunflower meals collected at the positive electrode of an electrostatic separator, presenting high contents in proteins and phenolic compounds. The objective was to make phenolics and proteins interact without addition of exogenous tannins. Treatment CH incubated a meal fraction:water mixture (1:2, w:w) for 48 h at 50 °C. Treatment FR incubated a meal fraction:water mixture (1:10, w:w) at pH 9.0 for 48 h at 4 °C. Microbial proteolysis on meal fractions were quantified during 24 h rumen batch fermentations with cellulose and starch as nitrogen-free energy sources. The net production of ammonia tended to be reduced by treatment FR mostly on rapeseed, corresponding to an 8% saving of rapeseed meal proteins degradable in the rumen. When untreated, the sunflower fraction decreased methane production by 50%, while treatments restored the fermentation pattern. Cold alkaline treatment could be considered to protect meal proteins from degradation by rumen micro-organisms.

Keywords: rapeseed meal / sunflower meal / self-tanning / ruminant

Résumé – Évaluation de deux méthodes d'auto-tannage de fractions de tourteaux de tournesol et de colza enrichies en protéines et composés phénoliques par mesure de la dégradabilité ruminale des protéines *in vitro*. Deux méthodes de tannage des protéines ont été évaluées pour contribuer au remplacement du formaldéhyde comme agent tannant des tourteaux destinés à l'alimentation des ruminants. Les matériaux expérimentaux étaient deux fractions de tourteaux de colza et tournesol collectées à l'électrode positive d'un séparateur électrostatique, présentant des teneurs élevées en protéines et en composés phénoliques. Le but était de faire interagir les composés phénoliques et les protéines sans addition de tanins exogènes. Le traitement CH a consisté à incuber un mélange tourteau/eau (1/2, poids/poids) pendant 48 h à 50 °C. Le traitement FR a consisté à incuber un mélange tourteau/eau (1/10, poids/poids) à pH 9,0 pendant 48 h à 4 °C. La protéolyse des fractions de tourteau par les microbes du rumen a été quantifiée lors de fermentations *in vitro* de 24 h avec de la cellulose et de l'amidon comme sources d'énergie sans azote. Le traitement FR a eu tendance à réduire la production nette d'ammoniac, principalement avec le colza, équivalant à la protection de 8 % des protéines de tourteau de colza dégradables dans le rumen. La fraction de tournesol non traitée a diminué la production de méthane de 50 %, cependant les traitements ont restauré le profil fermentaire. Le traitement alcalin à froid pourrait être envisagé afin de protéger les protéines du tourteau de la dégradation par les micro-organismes du rumen (the full text is available in French on https://www.ocl-journal.org/10.1051/ocl/2019051/olm).

Mots clés : tourteau de tournesol / tourteau de colza / auto-tannage / ruminant

☆ Contribution to the Topical Issue "Technological challenges in Oilseed crushing and refining / Défis technologiques de la trituration et du raffinage des oléagineux"

☆☆ The French version is available in "Supplementary Material".

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1 Introduction

Among the sources of dietary amino acids available to ruminants in Europe, rapeseed and sunflower cakes have been promoted for 50 years as alternatives to imported soybean meal (Grenet and Demarquilly, 1970; Richardson *et al.*, 1981). However, regardless of the origin of the meal, reducing the degradation of their proteins by rumen microorganisms has been a constant concern for nutritionists. Indeed, rumen deamination of amino acids is nutritionally disadvantageous to the animal. It is only partly offset by *de novo* synthesis of microbial protein and results in a partial loss of ammonia by excretion of urea. The lowering of the rumen degradability of proteins, initially obtained also by toasting (Grenet and Demarquilly, 1970), mainly uses tanning with formaldehyde, which reacts with about ten amino acids, mainly asparagine, glutamine, lysine and arginine, to form methylene bonds that cause protein reticulation (Barry, 1976; Verite *et al.*, 1977; Antoniewicz *et al.*, 1992). These bonds are then hydrolysed when transiting from the rumen content at neutral pH to the abomasum acid medium. Although this industrial process was considered safe for the animal because the free formaldehyde fraction is rapidly metabolised in the rumen to carbon dioxide (Puigserver *et al.*, 2004), growing social demand for more natural farming practices and the risks associated with the handling of formaldehyde during tanning have prompted the search for tanning agents of plant origin, in the continuity of pioneering work on chestnut wood hydrolysable tannins (Zelter *et al.*, 1970). Among the secondary metabolites tested, the phenolic compounds present in the rapeseed peel and meal have been studied for their action on rumen microbial metabolism and their ability to interact with proteins (Wischer *et al.*, 2013). However, the tanning power of rapeseed phenolic compounds is still poorly documented.

In addition, other ways of valorising rapeseed and sunflower meals, in particular as potential sources of bioactive molecules, have been studied (Hernandez-Jabalera *et al.*, 2015). Recently Laguna *et al.* (2018) evaluated several dry separation techniques on their ability to isolate protein-enriched or phenolic-enriched meal fractions in advance of more selective processes. They established that electrostatic separation made it possible to collect on the positive electrode fractions with protein and phenolic contents higher than in initial cakes by 50–55% and 80–100% for rapeseed and sunflower respectively. In fact, these fractions are suitable materials for studying proteins tanning ability using endogenous phenolic compounds. Indeed, using this potential self-tanning property of meals would limit the amounts of tannic extracts used during tanning or directly incorporated into the diet of ruminants.

Lowering the degradability of proteins by tannins involves various modes of interaction either covalent or non-covalent (Chu *et al.*, 2018). According to Hernandez-Jabalera *et al.* (2015), the hydrolysable tannins present in rapeseed meal can complex with proteins by reversible hydrogen bonds. The common tanning treatment consists of mixing the meal with at least twice its weight of water, keeping the mixture for at least 20 hours at room temperature or in an oven and drying it at a temperature not exceeding 80 °C (Zelter *et al.*, 1970). However, a second way of cold and alkaline tanning could exist. Recently, (Bongartz *et al.*, 2018) have shown that the alkaline treatment of sunflower meal induces the formation of chlorogenic acid quinones which react with the proteins of the meal. This phenomenon leads to the reduction of the simulated degradation of proteins by rumen microorganisms. Thus, the alkaline treatment increased rumen undegraded crude protein (RUP) values by factors ranging from about 3 (Kp = 0.08/h) to 12 (Kp = 0.02/h). Similar results were obtained in the laboratory (V. Solé-Jamault, personal communication). By subjecting the fraction of sunflower meal collected at the positive electrode to an extraction at pH 9 for 48 h, a crosslinking phenomenon was observed on electrophoresis gels suggesting a tanning of proteins.

Our study aims at quantifying the impact of two pretreatments – hot or cold and alkaline – on the rumen degradability of proteins in rapeseed and sunflower meal fractions enriched in proteins and phenolic compounds. An *in vitro* method makes it possible to attribute the variations in ammonia production to the deamination of the amino acids present in the meals fractions. The results were partially displayed as a poster at the International Rapeseed Congress held in Berlin in 2019.

2 Material and methods

2.1 Treatments of meal fractions

The self-tanning test was carried out on rapeseed and sunflower meal fractions harvested on the positive electrode of an electrostatic separator (TEP System, Tribo Flow Separations, Lexington, USA) and consisted of two steps, one pre-tanning, according to two methods described below, and an evaluation of this tanning by incubation in presence of rumen microbiota. The operating conditions of the electrostatic separation and the chemical composition of the two fractions are described by Laguna *et al.* (2018). Each fraction was divided into 3 batches: control (CT), cold pretreatment (CO) and heat pretreatment (HO). The CO pretreatment was a minimum 48 h maturation at 4 °C and pH 9.0 (by adding NaOH) in a closed container. The pretreatment HO was the mixture of the cake fraction with deionized water (1:2, w:w) followed by a maturation for at least 48 h at 50 °C in a closed container. The fractions were then freeze-dried.

2.2 Incubations

Three dry goats fitted with a rumen cannula provided the rumen content used as inoculum. They were housed in straw-bedded group pen in an approved experimental facility (no. B78-615-1002). They were fed a diet made of hay, barley, mineral and vitamin supplement in two meals a day. The experimental procedures were approved by the local Ethics Committee (COMETHEA approval number 12079) agreed by the French Ministry of Higher Education and Research (agreement code C2EA-45) and they were performed on animals by accredited personnel. The rumen contents collected (300 mL per animal) were immediately filtered through a 120 µm mesh nylon gauze and brought to the laboratory under a CO_2 atmosphere and in isothermal containers maintained at 37–40 °C. Three independent incubation series were done, each using the rumen contents of a given goat. In each series, incubations were made in

duplicate in 72 mL culture tubes containing 60 mg of cellulose, 40 mg of starch and 50 mg of one out of the three meal fractions (CT, CO or HO) along with 2 incubations containing no experimental substrate and called "blanks" in order to quantify the fermentation products of the substrates present in unknown amounts in the inoculum. Each culture tube was filled with 10 mL Simplex buffer solution at pH 6.7 ± 0.05 (Broudiscou and Lassalas, 2000), with 6 mL inoculum, flushed with CO_2 for 4 min to purge the head space from oxygen, tightly sealed and kept in a agitating water bath at 39 °C. Within each series, the culture tubes were randomly processed. Incubations were stopped 24 h (±5 min) later by cooling each tube at +4 °C for at least 30 min before any measurement or sampling was undertaken. Then the tube was processed as follows. The amount and composition of fermentation gases were immediately measured, then the fermentation broth pH was measured and the culture medium was sampled (1.2 mL stabilised with 0.3 mL ortho-phosphoric acid 25% v/v, in duplicate) to quantify the concentrations of short-chain fatty acids (SCFA) and ammonia. The samples were immediately stored at −21 °C until analysis. The inoculums were sampled following the same procedure to quantify their SCFA concentrations.

2.3 Analyses

Ammonia concentration in culture media and inocula was measured using a specific electrode (Broudiscou and Papon, 1994). In centrifuged culture media and inocula (10 min at 10 000 g), SCFA were separated by reverse-phase HPLC (4.6 × 150 mm, 3 µm C18 ultra-aqueous column, acetonitrile–phosphate buffer 50 mM pH 2.0 gradient) and their concentrations were quantified by spectrophotometry with a diode array detector (λ 210 nm, Jasco, Lisses France). The fermentation gases composition was measured by GC (Varian, MicroGC) (Broudiscou *et al.*, 2014).

2.4 Calculations and statistical analyses

The individual productions of SCFA, gases and ammonia from the fermentation of a given experimental substrate and used thereafter were calculated by difference between the amounts measured at the end of incubation in the tube containing the experimental substrate and those in the "blanks" of the same series.

The amount of hexoses fermented per tube (HF) was calculated using the formula proposed by Demeyer and Van Nevel (1975):

$$\mathrm{HF}=(\mathrm{PC2}+\mathrm{PC3})/2+\mathrm{PC4}+\mathrm{PC5}(\mu\mathrm{moles/tube}), \quad (1)$$

PC2, PC3, PC4 and PC5 being the amounts of acetate, propionate, butyrate and valerate produced per tube in 24 h respectively.

The specific production (PS) of each SCFA was calculated using the formula:

$$\mathrm{PS}=100\times \mathrm{producedamount/HF}(\mu\mathrm{moles}/100\,\mu\,\mathrm{moles\,HF}). \quad (2)$$

For each meal, the variables were subjected to an analysis of variance using the GLM procedure of the SAS 9.1 software (SAS/STAT®, 2000). The two means per pretreament were compared to the mean of controls using the Dunnett test. In order to limit the total first-order error to 0.05 for significant differences and 0.10 for trends, the significance and trend thresholds were set at $P < 0.025$ and $P < 0.05$.

3 Results and discussion

In rapeseed meal incubations (Tab. 1), the intensity of cellulose and starch fermentations, as measured by the HF variable, and their profile were normal, with a predominance of acetate production to the detriment of propionate and butyrate as well as an efficient methanogenesis which has strongly limited the presence of dihydrogen. No tanning method has changed, even in trend, the fermentative variables. Net productions of ammonia and isovalerate – produced by the decarboxylation of the carbon chains of leucine and isoleucine (Demeyer and Van Nevel, 1979) – were negative (Tab. 2), indicating that the fermentations associated with the experimental substrate induced an ammonia consumption by rumen microorganisms greater than its release by deamination. Cold pretreatment (CO) tended to increase this difference, whereas pretreatment at 50 °C (HO) did not have any effect even in trend. The effect of pretreatment CO can be attributed to a lower degradation of rapeseed meal proteins, clearly a self-tanning process, rather than a stimulation of microbial protein synthesis. Indeed, the final pH of the fermentation medium was greater than 6.0 and did not vary between treatments. ATP from fermentations was therefore used to provide the energy needed for microbial growth and not to maintain a neutral intracellular pH (Strobel and Russell, 1986). Our observations based on a biological test implementing rumen microbiota activity extend to rapeseed meal the conclusions of Bongartz *et al.* (2018) who measured by an enzymatic method a decrease in proteolysis in a CO treated sunflower meal.

In control incubations (CT) of sunflower meal (Tab. 3), the intensity of carbohydrate fermentations was normal. However, their profile has been characterized by reduced specific productions of acetate and methane, in favour of propionate and hydrogen disulfide, and accompanied by a slight accumulation of dihydrogen. The untreated sunflower fraction may have disrupted the metabolism of methanogenic archaea, this reduction of methanogenesis impacting the activity of cellulolytic and acetate-producing bacteria, *via* the regeneration of their NAD and NADP cofactors, which requires a low dihydrogen partial pressure. (Baldwin and Allison, 1983). In this context, the increase in specific productions of propionate and hydrogen disulfide can be interpreted as an alternative entrapment of dihydrogen. The polyunsaturated fatty acids contained in the sunflower seed – mainly linoleic acid – cannot be the cause of this decrease in methanogenesis because the meal subjected to electrostatic separation was previously delipidated to a final content of 2% DM (Laguna *et al.*, 2018). The inhibitory constituents are probably phenolic compounds (Mueller-Harvey, 2006). Eleven phenolic compounds, among which chlorogenic acid whose antibacterial activity has been observed in pure cultures (Lou *et al.*, 2011), were detected in the sunflower fraction (Laguna *et al.*, 2018) but their effects on methanogenic archaea

Table 1. Amount of hexoses fermented (HF) and specific productions of fermentation end-products from the experimental substrate containing the rapeseed meal fraction.

	HF	C2	C3	C4	CH_4	H_2	H_2S
	μmoles			moles/100 moles hexoses fermented			
Pr > F model	0.16	0.16	0.048	0.092	0.051	0.50	0.14
R^2	0.75	0.74	0.87	0.81	0.86	0.50	0.77
RSD	31.6	2.84	4.86	1.85	2.32	0.041	3.15
Source				Probability Pr > F			
Treatment	0.11	0.93	0.80	0.76	0.85	0.91	0.67
Inoculum	0.26	0.067	0.018	0.037	0.019	0.27	0.059
Treatment				Means per treatment ($N=3$)			
CO	446 (0.17)	91.5 (1.00)	63.3 (0.80)	22.6 (0.70)	30.9 (0.93)	0.078 (0.96)	25.8 (0.83)
HO	517 (0.75)	90.7 (0.93)	65.8 (1.00)	21.8 (0.97)	29.8 (0.96)	0.085 (0.87)	28.2 (0.89)
CT	499	91.4	65.6	21.5	30.3	0.070	27.1

Table 2. Net productions of ammonia (mg/tube) and isovalerate (μmoles/tube) from the rapeseed fraction and culture medium final pH.

	NH_3	IC5	pH 24 h
Pr > F model	0.0083	0.21	0.0003
R^2	0.95	0.71	0.99
SRD	0.092	8.97	0.025
Source		Probability Pr > F	
Treatment	0.011	0.60	0.82
Inoculum	0.0095	0.10	0.0001
Treatment		Means per treatment ($N=3$)	
CO	−0.622 (0.033)	−8.0 (0.71)	6.08 (0.93)
HO	−0.194 (0.20)	−5.7 (0.53)	6.08 (0.76)
CT	−0.339	−13.4	6.09

IC5: isovalerate; RSD: residual standard deviation; comparison of cold and hot pretreatments (CO and HO) with control (CT): the first-order errors are in brackets.

Table 3. Amount of hexoses fermented (HF) and specific productions of fermentation end-products from the experimental substrate containing the sunflower meal fraction.

	HF	C2	C3	C4	CH_4	H_2	H_2S
	μmoles			moles /100 moles hexoses fermented			
Pr > F model	0.011	0.034	0.029	0.30	0.058	0.42	0.025
R^2	0.94	0.89	0.90	0.64	0.85	0.56	0.91
RSD	14.8	4.36	4.10	1.59	5.57	0.91	10.2
Source				Probability Pr > F			
Treatment	0.0045	0.037	0.057	0.61	0.039	0.37	0.0092
Inoculum	0.14	0.043	0.022	0.16	0.13	0.39	0.66
Treatment				Means per treatment ($N=3$)			
CO	447 (0.0088)	89.1 (0.055)	64.1 (0.093)	23.4 (0.58)	31.0 (0.033)	0.10 (0.37)	19.5 (0.0065)
HO	533 (0.33)	91.3 (0.032)	61.6 (0.047)	23.5 (0.62)	28.3 (0.057)	0.10 (0.37)	36.7 (0.028)
CT	514	77.7	73.0	24.7	13.9	1.14	70.0

C2: acetate; C3: propionate; C4: butyrate; SRD: residual standard deviation; comparison of pretreatments (CO and HO) with control (CT): the first order errors are in brackets.

Table 4. Net productions of ammonia (mg/tube) and isovalerate (μmoles/tube) from the sunflower fraction and culture medium final pH.

	NH_3	IC5	pH
Pr > F model	0.019	0.0079	0.0059
R^2	0.92	0.95	0.95
SRD	0.183	4.54	0.052
Source		Probability Pr > F	
Treatment	0.020	0.072	0.26
Inoculum	0.026	0.0037	0.0022
Treatment		Means per treatment ($N = 3$)	
CO	0.15 (0.070)	−14.8 (0.064)	6.16 (0.51)
HO	0.88 (0.20)	−12.6 (0.11)	6.12 (0.20)
CT	0.59	−3.3	6.21

IC5: isovalerate; RSD: residual standard deviation; comparison of cold and hot pretreatments (CO and HO) with control (CT): the first-order errors are in brackets.

are not documented. CO pre-treatment slightly reduced the intensity of fermentations. More surprisingly, both pretreatments restored the fermentation profiles, characterized by active methanogenesis and a low presence of dihydrogen and close to those observed with rapeseed meal, suggesting that they inactivated the component of the meal fraction responsible for the partial inhibition of methanogens.

No pretreatment significantly changed the net production of ammonia (Tab. 4) and isovalerate. The hierarchy between fractions, however, was similar to that observed with rapeseed meal, CO pretreatment being associated with the lowest ammonia production, in agreement with the results of Bongartz *et al.* (2018).

In conclusion, only the cold and alkaline pretreatment tended to reduce the microbial proteolysis of the meal fractions (mainly for rapeseed meal), in a proportion too small, however, to present a nutritional advantage for ruminants. In addition, methanogenesis was reduced in the presence of the untreated sunflower fraction, suggesting the inhibitory action of a phenolic compound still to be identified.

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Supplementary Material

French version.
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